Aus der Medizinischen Klinik A
und der Abteilung Zytopathologie
(Direktor: Prof. Dr. Schneider, Prof. Dr. Dr. Pfitzer)

Ploidy pattern of megakaryocytes in patients with metastatic tumors with and without paraneoplastic thrombosis and in controls

Dissertation
zur Erlangung des Grades eines
Doktors der Medizin
Der Medizinischen Fakultät
der Universität Düsseldorf

vorgelegt von:
Joseph Stöckler

1983

Aus der Medizinischen Klinik A
und der Abteilung Zytopathologie
(Direktor: Prof. Dr. Schneider, Prof. Dr. Dr. Pfitzer)

Ploidy pattern of megakaryocytes in patients with metastatic tumors with and without paraneoplastic thrombosis and in controls

Dissertation
zur Erlangung des Grades eines
Doktors der Medizin
Der Medizinischen Fakultät
der Universität Düsseldorf

vorgelegt von:
Joseph Stöckler

1983

Ploidy pattern of megakaryocytes in patients with metastatic tumors with and without paraneoplastic thrombosis and in controls

J. Stöckler

Gedruckt mit Genehmigung der Medizinischen Fakultät der Universität Düsseldorf

Der Dekan
der Medizinischen Fakultät
der Universität Düsseldorf

gez.: Prof. Dr. Hopf

Referent

gez.: Prof. Dr. Dr. Pfitzer

Korreferent

gez.: Prof. Dr. Göbel

Introduction

Coagulation disorders are a well-known phenomenon in patients with malignant tumors. Many investigations have been performed which suggest an involvement of the haemostatic system at various levels in malignancy (1). Aside from DIC, thrombotic events are the most common complication in malignancy, occurring in more than 50 % of patients (2). However, the reasons are still not clearly understood.

There is no doubt that thrombocytes are not only a main factor in thrombogenesis. Interactions between platelets and tumor cells are likely to play an important role in cancer cell dissemination, and platelet aggregation inhibitors have been used to reduce the number of metastases (3). As we know from numerous investigations (4, 5, 6) the platelet population is not homogenous, and platelets vary in density as well as in the number of mitochondria, and granules, and the size of the surface connected canalicular system. There is evidence that this heterogeneity depends on the different ploidy of the megakaryocytes, they are derived from, rather than on aging during their life span (7, 8).

As in other polyploid human tissues like heart or liver, a certain variability in the degree of ploidy is to be expected in megakaryocytes. An interesting question is whether there are changes in the megakaryocyte ploidy pattern of patients with metastatic tumors with or without paraneoplastic thrombosis in comparison to healthy controls Very little is known about megakaryocytes in patients with malignant tumors. Their thrombogenic role in malignancy is so far a matter of speculation.

Materials and Methods

Bone marrow aspirates from 15 patients with histologically confirmed metastatic tumors and paraneoplastic thrombosis were investigated, six male and nine female. The ages ranged from 38 to 77 years with an average of 62 years. Nine of the patients had adenocarcinoma, three squammous cell carcinoma, two malignant lymphoma and one hepatocellular carcinoma.

In another 15 patients with metastatic tumors but without pre- or postmortem evidence of thrombosis, bone marrow aspiration was performed. Six were male and eight female. The ages ranged from 24 to 78 years with an average of 62 years. The histological types of the tumors were: seven adenocarcinomas, five squammous cell carcinomas, two small cell carcinomas and one hepatocellular carcinoma.

Bone marrow aspirates from 10 hospitalized subjects were investigated as controls. These patients had no pathological blood count findings, especially regarding platelets, and no disorder in the common coagulation tests. There was no evidence of haematologic, renal, hepatic or oncologic disease. Six were male and four female.

The ages ranged fom 19 to 76 years with an average of 55 years. In three patients who died of myocardial infarction within one day of admission we obtained additional bone marrow aspirates immediately after death and every six hours up to 30 hours post mortem for DNA measurement. During this time the corpses were preserved at 4° C.

In ten cases with paraneoplastic thrombosis bone marrow aspirates were obtained during the first 12 hours post mortem. In all these patients deep vein thrombosis of the legs and in four of them additional pulmonary embolism were found at autopsy. The age of the thromboses ranged from two days to about six weeks. Five of these patients had multiple thrombi of different age. The five remaining patients were punctured during hospitalization. Three of them had deep vein thromboses of the legs which were diagnosed clinically and by phlebography; two patients suffered from thrombophlebitis migrans.

All bone marrow smears were fixed with 96 % ethanol for 30 minutes. The stages of maturity were determined in vitally obtained bone marrow aspirates after dyeing by Pappenheim stain.

The stages of maturity were distinguished according to cytoplasmic criteria:

Type I	Megakaryoblast: completely basophilic cytoplasm
Type II	Promegakaryocytes: basophilic cytoplasm with faint azurophilic granulation
Type III	mature Megakaryocyte: strongly azurophilic granulation, no basophilia, cytoplasm sometimes disintegrating into platelets.

In order to detect as many megakaryocytes as possible, Pappenheim staining was also carried out in postmortally obtained bone marrow aspirates, though maturity could no longer be assessed. For recognition after Feulgen staining the coordinates of every evaluated megakaryocyte were noted. After the classification of megakaryocyte maturity the smears were decolorised in 50 % ethanol for 10 minutes and then restained by the Feulgen reaction.

The DNA content of Feulgen-stained megakaryocytes was measured with the Zeiss-Scanning Cytophotometer using monochromatic light (570 nm). DNA measurement of lymphocytes was used as internal control. To avoid technical mistakes all megakaryocytes of one slide which were not overlapping with other nuclei were measured. The number of evaluated and measured megakaryocyte nuclei per patient ranged from 1oo to 442 with an average of 155.

Post mortem DNA measurement

DNA histograms obtained immediately after death and six and 12 hours post mortem showed no significant differences in ploidy pattern (Fig. 1). This implies that DNA values do not change within the first 12 hours post mortem. The earliest alterations became evident after 18 hours in one patient and after 24 hours in the two remaining patients. After this time the limits between the ploidy classes in the DNA histograms become effaced, and after 24 hours no clearly separate DNA classes can be differentiated.

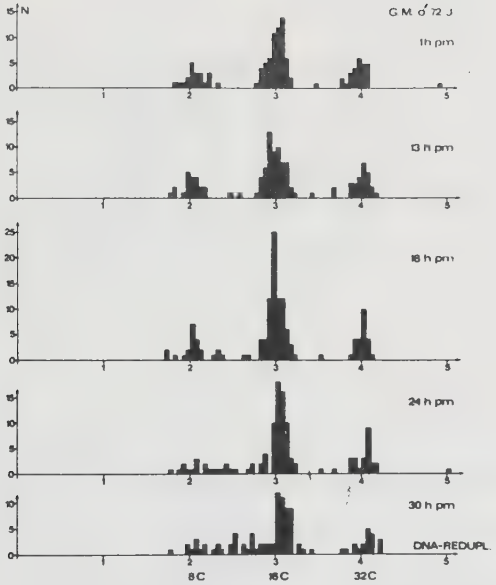

Fig. 1 Postmortal DNA-histograms of megakaryocytes as follow-up from 1 to 30 hours of one patient.

Results

Megakaryocyte maturity stages and the ploidy patterns of the control group.

The distribution of maturity stages among the subjects of the control group does not vary significantly ($p > 0.2$). For this reason all evaluated megakaryocytes of this group are taken together. Megakaryoblasts (Type I) comprised 18.0 % (15.0-20.6 %) and were as frequent as the promegakaryocytes (Type II) with 17.0 % (15.4-17.8 %), while the mature megakaryocytes (Type III) predominated with 65.1 % (61.7-67.3 %). The relation between Type I : Type II : Type III was 1 : 1 : 3.7 and the average maturity stage is therefore 2.47.
The DNA histograms of the 10 probands forming the control group are characterized by three sharply separated peaks with a 4-, 8-, and 16-fold DNA content of the diploid lymphocytes. Therefore they can be classified as 8c, 16c and 32c megakaryocytes. Since the DNA histograms of the controls are uniform, an average ploidy pattern for the megakaryocytes of all 10 probands can be calculated (Fig. 2). 8c megakaryocytes are with 18.9 % (15.9-23.7 %) only slightly less frequent than 32c megakaryocytes with 24.1 % (20.5-26.2 %). 16c megakaryocytes form the main bulk with 56.4 % (49.5-62.6 %). Only very few 64c megakaryocytes, 0.4 % (0-1 %) were detected. Forming an index value by calculating the average number of DNA reduplications an average ploidisation index of 3.06 is obtained for the controls.

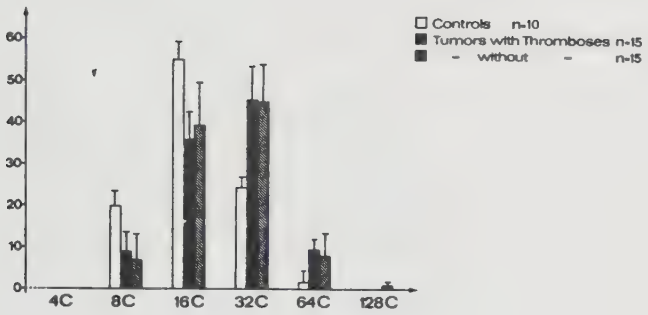

Fig. 2 DNA classes of megakaryocytes in controls and tumor patients with or without thromboses.

Beside the main peaks of 8c, 16c and 32c a few values are situated exactly between the main ploidy classes. They have a DNA content of 12c, 24c and 48c and amount to 1.6 % (o.5-3.4 %) of the total.

Patients with metastatic tumors and paraneoplastic thrombosis

The distribution of the maturity stages in the five tumor patients studied pre mortem does not differ from the maturity distribution of the control group. Megakaryocytes of Type I : Type II : Type III are found at the rate of 1 : 1 : 3.5-4 and the average maturity stage amounts to 2.46-2.5o. In the DNA histograms of the 15 patients with paraneoplastic thrombosis the megakaryocytes also form sharply separated peaks at different ploidy values. However, in contrast to the control group, all 15 patients show a shift to higher ploidy values (Fig. 2).
32c megakaryocytes form the main portion with 45.o±7.7 % whereas 8c nuclei are reduced to 9.o±4.6 % and 16c nuclei to 36.9±6.7 %. 64c nuclei, which are found only sporadically in normal subjects achieve 9.2±5.o % and are as frequent as 8c nuclei. In one case even a 128 nucleus is measured. Calculating the average number of DNA reduplications as an index value yields the highly significant value of 3.54 in comparison with 3.o6 for the control group (Fig. 3).

In one patient who had received chemotherapy until 8 weeks before the bone marrow study, 4.2 % of the megakaryocytes contain 4c nuclei by DNA measurement. Nevertheless the average ploidy index in this patient (3.28) is significantly higher than in the control subjects. Thus all 15 patients with paraneoplastic thrombosis show a significant shift ($p < 0.0001$) to higher ploidy values, which is somewhat less marked in that patient under cheomtherapy.

Patients with metastatic tumors without thrombosis

In this group we find exactly the same shift to higher ploidy values (Fig. 2). The normally dominant 16c class is reduced to 39.5±10.1 % and 8c nuclei to 6.8±6.1 %. The 32c class doubles to 44.9±9.2 % while 64c megakaryocytes attain 8.0±5.6 % and show a 20-fold increase in comparison to the controls. Some 128c nuclei are also seen here. The average ploidisation index of 3.56 is essentially the same as in the patients with paraneoplastic thrombosis (Fig.3).

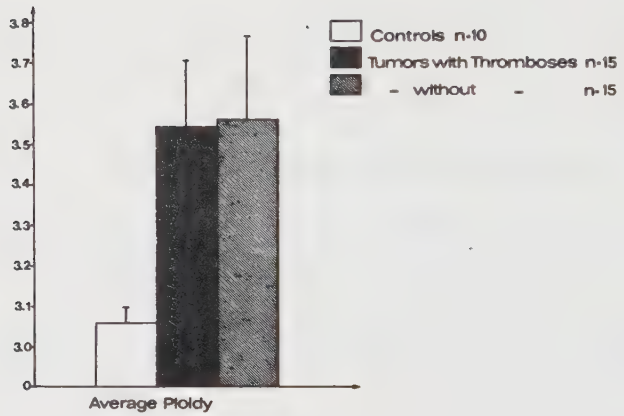

Fig. 3 Average ploidy of the megakaryocytes in 10 control cases and 15 tumor patients with and 15 without thromboses.

Discussion

Several authors (9, 1o) have investigated megakaryocyte ploidy values in myeloproliferative disorders such as polycythaemia vera, chronic myeloid leukemia and idiopathic thrombocytosis, in which coagulation disorders are a cardinal symptom. In idiopathic thrombocytosis and polycythaemia vera a majority of cases showed a shift of the megakaryocytes to higher ploidy values. In these myeloproliferative disorders, however, megakaryocytes are involved in the neoplastic process itself and atypical megakaryocytes with altered cell shapes and bizarre nuclei are observed (11).

All our patients with metastatic tumors show a significant shift to higher ploidy values in megakaryocytes, independent of whether these patients suffer from thrombosis or not. We thus conclude that paraneoplastic thrombosis alone cannot be the explanation. Metastasic and tumor, however, seem to be a basic requirement.

Since no indication exists that megakaryocytes of patients with solid malignant tumors are directly involved in the malignant process, changes in ploidy distribution should be a reaction to the tumor growth or to alterations mediated by the tumor or its metastases.

It is suggested that there is a "mild" form of DIC in all patients with metastatic tumors which maintains a compensated consumption of platelets, fibrinogen and other coagulation factors. This hypercoagulable state could easily lead to thrombotic or haemorrhagic complications (12) in the presence of additional factors such as operation or trauma. The results of Hasiba (13) who found manifest DIC in about 40 % of tumor patients, support this assumption and in subtile investigations of the coagulation system alterations in at least one parameter were found in 96 % of all investigated patients with inoperable lung cancer (14). This perpetual subclinical hypercoagulable state with progressive consumption of clotting factors could actually be due to the well known thromboplastic activities (15) or the cancer procoagulant activities demonstrated in cancer cells, particularly those of adenocarcinoma (16). Furthermore, tumor cells are able to clump thrombocytes by releasing plasma membrane vesicles which bind to the platelets. This process is mediated by the first steps of the complement system and leads finally to platelet aggregation. Although there are very few kinetic studies on platelet turnover in malignancy there are multiple indications for an increased platelet consumption (17, 18).

As is known from the studies of Penington (19), an increased consumption of platelets is primarily compensated by an increase in the cytoplasm of the megakaryocytes together with a shift to higher values. Our results could therefore be interpreted as the response of megakaryocytes to an increased demand of platelets.

On the other hand, the well known fact that malignant tumors are often accompanied by thrombocytosis is not explained by this hypothesis. All our patients had either normal or evaluated peripheral platelet counts. Thrombocytosis is, however, regarded by some authors (12) as overcompensation for a continuous platelet consumption.

There is a strong evidence that a thrombopoietin-like activity is responsible for the achievement of higher ploidy levels in megakaryocytes (2o). The level of thrombopoietin seems to vary inversely with the number of circulating platelets. Thus, even if an increase platelet turnover is postulated, the specific stimulus for thrombopoietin production is lacking in patients having normal or increased platelet counts.

Thus other possible explanations for the ploidy shift to the right must be considered. If a mitogenic or growth factor with thrombopoietin activity produced by the tumor itself is postulated, one could expect the same effect on the megakaryocytes. An increased platelet count could then be explained by a disturbed feedback mechanism between the amount of platelets and the thrombopoietin level, due to autonomous production of tumor thrombopoietin-like activity. This would not only lead to higher ploidy values but also to an alteration in heterogenity of peripheral platelets with an increase of the platelet fraction with lower density. Perhaps a thus altered platelet activity could favour, but not necessarily lead to, thrombotic complications.

Further questions need to be answered: For instance, can thrombosis without malignancy lead to higher ploidisation of the megakaryocytes ? Or are there diseases associated with a shift to lower ploidy values in the megakaryocyte DNA pattern ?

Summary

In order to measure megakaryocyte DNA content in a greater number of well-defined patients, the use of bone marrow aspirates obtained post mortem is a basic requirement. We could show that the distinction between the ploidy classes in DNA histograms is possible until 18 hours post mortem. Thus bone marrow aspirates obtained up to 12 hours after death can be expected to give reliable results.

Megakaryocytes of the following patient groups were studied: 15 patients with metastatic tumors and paraneoplastic thrombosis, 15 patients with metastatic tumors without paraneoplastic thrombosis and 10 controls. An increased ploidisation of the megakaryocytes was found in all 30 patients with metastatic tumors, independently of whether these patients suffer from thrombosis or not. Higher megakaryocyte ploidisation, however, is correlated with a larger cytoplasmic mass of megakaryocytes, which leads to an increased platelet production. Besides an overcompensation for possibly increased platelet consumption, a mitogenic or thrombopoietin-like factor produced by the tumor itself must be considered.

References

1. Donati, M.B.; Poggi, A.; Semeraro, N.:
Coagulation and malignancy in Poller, pp 227-26o
(Churchill Livingstone Edinburgh, London, Melbourne,
New York, 1981). Recent advances in blood coagulation Vol 3.

2. Ambrus, J.L.; Ambrus, C.M.:
Blood coagulation in neoplastic disease; in Gastpar,
Onkohämostaseologie, pp. 167-193
(Schattauer, Stuttgart 1976)

3. Gastpar, H.: Beeinflussung der Metastasierung durch
Hemmung der Thrombozytenaggregation. Beitr. Onkol. 13:
29o-3o6 (Karger, Basel 1982)

4. Karpatkin, S.: Heterogeneity of human platelets.
I. Metabolic evidence suggestive of young and old platelets.
J. Clin. Invest. 48: 1o73-1o82 (1969)

5. Karpatkin, S.: Heterogeneity of human platelets.
II. Functional evidence suggestive of young and old
platelets.
J. Clin.Invest. 48: 1o83-1o87 (1969)

6. Penington, D.G.; Streatfield, K.:
Heterogeneity of megakaryocytes and platelets.
Ser. Haematologica 8 (1): 22-48 (1975)

7. Penington, D.G.; Lee, N.Y.T.; Roxburgh, A.E.; McGready, J.E.:
Platelet density and size: the interpretation of heterogeneity.
Br. J. Haemat. 34: 365-376 (1976)

8. Penington, D.G.; Streatfield, K.; Roxburgh, A.E.:
Megakaryocytes and the heterogeneity of circulating
platelets.
Br. J. Haemat. 34: 639-653 (1976)

9. Lagerlöf, B.: Cytophotometric study of megakaryocyte ploidy in polycythaemia vera and chronic granulocytic leukaemia.
A. Cytol. 16: 24o-244 (1972)

1o. Queißer, W.; Weidenauer, G.; Queißer, U.; Kempgens, U.; Müller, U.: Megakaryocyte polyploidization in myeloproliferative disorders.
Blut 32: 13-2o (1976)

11. Georgii, A.; Vykoupil, K.F.; Thiele, J.:
Chronic megakaryocytic granulocytic myelosis - CMGM. Virchows Arch. A. Path. Anat. and Histol.389: 253-268 (198o)

12. Deutsch E.; Blutgerinnungsstörungen bei nicht-haematologischen malignen Erkrankungen.
Beitr. Onkol. 13: 176-289 (1982)

13. Hasiba, U.; Lewis, J.H.; Spero, J.A.:
Coagulation patterns in patients with solid tumors.
Thromb. Haemostasis 42: 143 (1979)

14. Hagedorn, A.S.; Bowie, E.J.W., Elveback, L.R.; Owen, C.A.: Coagulation abnormalities in patients with inoperable lung cancer.
Mayo Clin.Proc. 49: 647-653 (1974)

15. Gralnick, H.R.: Cancer cell procoagulant activiy; in Donati, Davidson, Garattini, Malingnany and the hemostatic system. pp. 57-63 (Raven Press, New York 1981)

16. Semeraro, H.; Donati, M.B.:
Pathways of blood clotting initiation by cancer cells; in Donati, Davidson, Garattini, Malignancy and the hemostatic system, pp. 65-81 (Raven Press, New York 1981)

17. Harker, L.A.; Slichter, S.J.:
Platelet and fibrinogen consumption in man.
N. Engl. J. Med. 187: 999-1oo5 (1972)

18. Abrahamsen, A.F.: Platelet turnover in metastatic cancer and the effect of platelet aggregation inhibitors.
Z. Krebsforsch. 86: 1o9-111 (1976)

19. Penington, D.G.; Olsen, T.E.:
Megakaryocytes in states of altered platelet production; cell numbers, size and DNA content.
Br. J. Haemat. 18: 447-463 (1970)

2o. Odell, TT.; Murphy, J.R.: Jackson, C.W.:
Stimulation of megakaryopoesis by acute thrombocytopenia in rats.
Blood 48: 765-775 (1976)

Lebenslauf

19.01.1958	geboren in Essen als Kind des Realschullehrers Josef Stöckler und der Grundschullehrerin Ruth Stöckler, geb. Kleine
1964 - 1967	Besuch der Josefschule in Essen (Städt. Volksschule) bis zum Abschluß der 4. Schulklasse
1967 - 1976	Besuch der Humboldtschule Essen (Städt. Gymnasium) mit dem Abschluß der Allgemeinen Hochschulreife
1977 - 1983	Medizinstudium an der Universität Düsseldorf mit dem Abschluß der Ärztlichen Prüfung
07.03.1980	Heirat mit Annette Stöckler, geb. Kampmann

Copy + Sofortdruck GmbH

Bilker Allee 173 Telefon 0211/31 70 20

Copy + Sofortdruck GmbH
Bilker Allee 173 Telefon 0211/317020